SUPPLEMENTAL METHODS

Animal Preparation

Female domestic crossbreed swine weighing 42.2±0.4 kg were sedated with 25 mg/kg ketamine IM. Animals were subsequently intubated, positive-pressure ventilated with room air (Dräger 4A, Narkomed, Telford, PA), and anesthetized with isoflurane (1.0-2.0 Vol%). Tidal volume (8-12 ml kg-1) and FiO_2 were administered to maintain end-tidal CO_2 ($ETCO_2$) between 38-42 mmHg and O_2 saturation ≥ 90% (CO_2SMO Plus, Novametrix Systems, Wallingford, CT). Esophageal temperature was measured and maintained via a heating blanket (Bair Hugger, Augustine Medical, Eden Prairie, MN) at 37±0.5°C throughout surgical preparation.

The left femoral artery and right external jugular vein were cannulated percutaneously with 8 French sheaths and micromanometer tipped catheters (Mikro-Tip Transducer, Millar Instruments, Houston, TX) were inserted to continuously measure aortic and right atrial pressures, respectively. Echocardiograms (ECG), $ETCO_2$, transcutaneous O_2 saturation, and temperature were continually recorded (Biopac Systems, Goleta, CA and LabView, National Instruments, Austin, TX). An IV bolus of heparin (5000 units) was administered once catheters were in place. An arterial blood gas was drawn after surgical preparation (Instrumentation Laboratory, Bedford, MA).

Mitochondrial Isolation

At the conclusion of the experimental protocol, mitochondria were isolated at 4°C via differential centrifugation from the left ventricle of the heart and brain as described previously(1,2). All reagents were purchased from Sigma (St. Louis, MO).

Cardiac mitochondria: The heart was removed via lateral thoracotomy immediately after chest compression were discontinued. Three g of left ventricular tissue was placed in ice-cold isolation buffer (200 mmol mannitol, 50 mmol sucrose, 5 mmol KH2PO4, 5 mmol MOPS, 1 mmol EGTA, 0.1% BSA, pH adjusted with KOH to 7.15), minced, and homogenized in the presence of 5 U ml^{-1} Bacillus Licheniformis. The suspension was centrifuged for 10 minutes at 8,000 g. The pellet was resuspended in 25 ml isolation buffer, followed by centrifugation for 10 minutes at 750 g. The resulting supernatant was centrifuged for 10 minutes at 8,000 g, and the pellet resuspended in 800 μl isolation buffer and stored on ice. Protein concentration was determined via the Bradford method(3).

Brain mitochondria: The brain was removed while the animal was supine with ongoing chest compressions to maintain cerebral perfusion. An incision was made in the scalp and the skull was removed with a bone saw (810 Autopsy Saw, Stryker, Kalamazoo, MI). The whole brain was removed and placed in ice-cold saline. Both hippocampi and the cerebellar vermis were combined with parietal cortex to a total of 8 g, representing a globally distributed sample of brain comprising regions sensitive to ischemia and required for executive function. This combination was placed in ice-cold isolation buffer,

minced, and homogenized via Dounce-style glass homogenizer (885300-0100, Kimble Chase, Rockwood, TN). The suspension was centrifuged for 3 minutes at 1,330 g. The supernatant was saved, and the pellet was suspended in isolation buffer and spun at 1,330 g for 3 minutes. The resulting supernatants were combined and spun for 10 minutes at 21,000 g. The pellet was suspended in isolation buffer containing 15% Percoll (GE Healthcare Bio-Sciences, Pittsburgh, PA). This solution was layered on top of a Percoll density gradient containing 40% and 24% Percoll in isolation buffer. The gradient was spun at 30,700 g for 9 minutes to isolate mitochondria from synaptosomes. The mitochondrial fraction was identified as the fraction between the 24% and 40% Percoll, and was twice suspended in isolation buffer and spun at 16,900 g and 6,900 g for 10 minutes each to remove excess Percoll. The resulting pellet was resuspended in 400 μl isolation buffer. Protein concentration was determined via the Bradford method(3).

Mitochondrial Function Tests

Respiration: Experiments were conducted at 25°C with mitochondria suspended (0.5 mg ml^{-1}) in experimental buffer (130 mmol KCl, 5 mmol K_2HPO_4, 5 mmol MOPS, 0.1% BSA, pH adjusted to 7.15 with KOH). Mitochondrial O_2 consumption was measured with a Clark-type O_2 electrode (Model 1302, Strathkelvin Instruments, North Lanarkshire, Scotland) in a water-jacketed 550-µl chamber (Model MT200A, Strathkelvin Instruments) monitored by an O_2 meter (Model 782, Strathkelvin Instruments). State (S) 2 respiration was initiated 60 seconds after sealing the chamber by adding 10 mM of the

complex I substrates pyruvate and malate or a combination of 10 mM complex II substrate succinate with 0.5 µM complex I blocker rotenone. Addition of 250 µM adenosine diphosphate (ADP) at 150 seconds initiated S3 respiration, until complete phosphorylation of ADP to ATP led to S4 respiration (Fig 1a). Chamber O_2 concentration in µM was monitored for 60 seconds after S4 respiration was achieved or until the O2 concentration was 0. Respiratory control index (RCI), a measure of the coupling of oxygen consumption to ATP generation, was calculated as the ratio of the rate of S3 to S4 respirations. All individual results are the average of duplicate runs.

ATP synthesis: The rate of mitochondrial ATP synthesis was determined by chemiluminescence (Glomax 20/20, Promega, Madison, WI) utilizing the reaction of firefly luciferase and luciferin with ATP. Each reaction contained 30 µM ADP, 10 µg ml^{-1} mitochondria, 0.1 mg ml^{-1} luciferin, and 1.25 µg ml^{-1} luciferase dissolved in experimental buffer. The reaction was measured for 100 seconds after addition of either 5 mM pyruvate/malate or succinate. To obtain background activity, each measurement is repeated in the presence of ATP synthase inhibitor oligomycin (1 μg ml^{-1}). Data from measurements in the presence of oligomycin are subtracted from those obtained in the absence of oligomycin. A standard curve was obtained from known ATP concentrations to calculate the rate of ATP synthesis.

CRC: Mitochondria were suspended (0.5 mg ml^{-1}) in experimental buffer inside a cuvette-based spectrofluorometer (QuantaMaster 800, Photon Technology

International, Edison, NJ) containing 10 mM of the complex I substrates pyruvate and malate or 10 mM of the complex II substrate succinate with 0.5 μM of the complex I blocker rotenone (Fig 1b). Extramitochondrial calcium concentration ($[Ca^{2+}]_{em}$) was monitored using the fluorescent probe CaGreen-5N hexapotassium salt (C3737, Life Technologies, Carlsbad, CA) at excitation and emission wavelengths of 510 and 531 nm, respectively. After a 1-min stabilization period, $CaCl_2$ (5 mM) was infused at a rate of 30 µl min^{-1} until $[Ca^{2+}]_{em}$ reached a steady state (equilibrium between Ca^{2+} infusion and mitochondrial Ca^{2+} uptake). $CaCl_2$ was continuously infused until a rapid increase in $[Ca^{2+}]_{em}$ was observed indicating release of mitochondrial Ca^{2+} from opening of the mitochondrial permeability transition pore (mPTP). CRC was quantified as the amount of $CaCl_2$ infused until mPTP opening.

Electron Spin Resonance

Production of reactive oxygen species (ROS) was assessed with electron spin resonance (ESR) in tissue frozen in liquid nitrogen immediately upon harvest. ROS species such as superoxide and hydrogen peroxide react with mitochondrial Fe_4-S_4 clusters contributing to aconitase inactivation and resulting in a characteristic aconitase radical with g-factor 2.002(4). Production of ROS was quantified by accumulation of aconitase free radical measured by ESR in frozen tissue using quartz Dewar and liquid nitrogen. The ESR spectrum of aconitase radical partially overlaps with other radical species. Subtraction of background ESR spectra revealed a clear aconitase free radical ESR spectrum (Fig S1c). The position of this spectrum (2.002 g-factor), line-width (8

Gauss), and high temperature and microwave power dependence confirmed identification as aconitase radical(5). The ESR signals of tissue background bioradicals did not change significantly with treatments (Fig S1d-g). ESR spectra were recorded (EMX ESR spectrometer, Bruker Biospin Corp., Billerica, MA) with super high Q microwave cavity (Suprasil Nitrogen Dewar Flask, Wilmad-Labglass, Vineland, NJ). The ESR settings were as follows: field sweep, 400 Gauss; microwave frequency, 9.43 GHz; microwave power, 20 milliwatts; modulation amplitude, 5 Gauss; sweep time, 80 seconds.

Figure Legend

Figure S1. Mitochondrial function tests. Representative tracings from closed-cell respirometry (a), calcium retention capacity (CRC, b), and electron spin resonance (ESR, c-f). Respiratory control index was calculated as the ratio of State 3 to State 4 respiration. CRC was quantified as the amount of calcium added to the reaction cuvette prior to the steepest slope of extramitochondrial calcium fluorescence. The amplitude of oxidized aconitase was determined after subtracting background bioradicals from ESR spectra (g).

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